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Site-directed mutagenesis and functional analysis of an active site tryptophan of insect chitinase

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Abstract

Chitinase is an enzyme used by insects to degrade the structural polysaccharide, chitin, during the molting process. Tryptophan 145 (W145) of *Manduca sexta* (tobacco hornworm) chitinase is a highly conserved residue found within a second conserved region of family 18 chitinases. It is located between aspartate 144 (D144) and glutamate 146 (E146), which are putative catalytic residues. The role of the active site residue, W145, in *M. sexta* chitinase catalysis was investigated by site-directed mutagenesis. W145 was mutated to phenylalanine (F), tyrosine (Y), isoleucine (I), histidine (H), and glycine (G). Wild-type and mutant forms of *M. sexta* chitinases were expressed in a baculovirus-insect cell line system. The chitinases secreted into the medium were purified and characterized by analyzing their catalytic activity and substrate or inhibitor binding properties. The wild-type chitinase was most active in the alkaline pH range. Several of the mutations resulted in a narrowing of the range of pH over which the enzyme hydrolyzed the polymeric substrate, CM-Chitin-RBV, predominantly on the alkaline side of the pH optimum curve. The range was reduced by about 1 pH unit for W145I and W145Y and by about 2 units for W145H and W145F. The W145G mutation was inactive. Therefore, the hydrophobicity of W145 appears to be critical for maintaining an abnormal pK_a of a catalytic residue, which extends the activity further into the alkaline range. All of the mutant enzymes bound to chitin, suggesting that W145 was not essential for binding to chitin. However, the small difference in K_m's of mutated enzymes compared to K_m values of the wild-type chitinase towards both the oligomeric and polymeric substrates suggested that W145 is not essential for substrate binding but probably influences the ionization of a catalytically important group(s). The variations in k_{cat}'s among the mutated enzymes and the IC_{50} for the transition state inhibitor analog, allosamidin, indicate that W145 also influences formation of the transition state during catalysis. Published by Elsevier Science Ltd.

Keywords: Chitinase; Tobacco hornworm; Site-directed mutagenesis; Active site tryptophan; Hydrophobicity; pH optimum; Glycosyl hydrolase; Insect; Molting; Chitin, Lepidoptera, Sphingidae; Enzyme mechanism; Hydrolysis; Binding; *Manduca sexta*

1. Introduction

Chitinases belonging to family 18 glycosyl hydrolases (Henrissat and Davies, 1997; Coutinho and Henrissat, 1999) have been isolated from a wide variety of sources including bacteria, yeast and fungi, nematodes, arthropods and mammals including humans (Nagano et al., 2001). All of these enzymes have two highly conserved regions that are involved in substrate binding and/or catalysis (Kramer and Muthukrishnan, 1997; Fukamizo, 2000). In particular, amino acid residues in the second conserved region, which has the consensus sequence (F/L)DG(L/I)DLD(W/I)EYP, have been implicated as important for catalysis from site-directed mutagenesis studies of genes encoding chitinases from several microbial sources (Watanabe et al., 1993, 1994; Thomas et al., 2000; Lin et al., 1999; Papanikolau et al., 2001; Lu et al., 2002). In these studies, the focus was on the acidic residues, namely glutamic acid and aspartic acid, which have been postulated to play roles similar to active site residues E35 and D52 of chicken lysozyme,

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respectively (Sinnott, 1990). The tryptophan residue found between these two catalytically important residues in *Manduca sexta* chitinase is completely conserved in all of the insect and nematode chitinases so far studied (Huang et al., 2000). In plant chitinases, this residue is an isoleucine with the exception of one tobacco chitinase (Zhang, 2000). In microbial chitinases, this residue is mostly W, although there are examples of the occurrence of I, L, F or Y at this position (Watanabe, 1995). These observations indicate that the presence of a hydrophobic residue may be critical for maintaining the activity of these enzymes. In a previous effort to understand the importance of the tryptophan in this conserved sequence, we had carried out site-directed mutagenesis of the gene for *M. sexta* chitinase, which involved the substitution of this residue with either phenylalanine or glycine (Huang et al., 2000). These studies demonstrated that replacement of W145 with glycine resulted in complete loss of activity, whereas the W145F mutant enzyme had a reduced activity and a narrower pH range over which the enzyme was active.

Studies on the role of tryptophan residues in other glycosyl hydrolases, including lysozyme and some plant chitinases, have indicated that this amino acid can play different roles in substrate binding, such as by hydrogen-bonding with substrate via the indole NH- group and by a stacking interaction with sugar rings of the substrate (Cheetham et al., 1972; Maenaka et al., 1998). Crystallographic studies of the plant chitinase hevamine-allosamidin complex and of a substrate-chitinase complex from the fungus, *Coccoides immitis*, have also revealed stacking of tryptophan residues over sugars in the subsites of the substrate-binding pocket (van Scheltinga et al., 1995; Hollis et al. 2000). Additionally, W539 of *Serratia marcescens* chitinase (another family 18 chitinase) has been postulated to participate in catalysis by forcing a substrate ring distortion to make the sugar at the -1 site assume a boat configuration (Brameld and Goddard, 1998a,b). To obtain a better understanding of the enzymatic role of tryptophan in conserved region II of *M. sexta* chitinase, we have carried out site-directed mutagenesis of W145 with several less polar residues, as well as a polar amino acid, histidine. The enzymatic and chitin-binding properties of these active site-modified enzymes are reported in this paper.

2. Materials and methods

2.1. Site-directed mutagenesis

The unique site elimination (U.S.E.) mutagenesis kit (Amersham Pharmacia Biotech, Piscataway, NJ) was used for site-directed mutagenesis of the 1.8 kb EcoRI fragment containing the coding region of *M. sexta* chitinase in pBluescript (Kramer et al., 1993). To eliminate the two PvuI sites in pBSKS (+) plasmid, two selection primers were designed as follows (Pharmacia protocol): PvuI A: 5′-G T T G G G A A G G **G C A A T T** G G T G C G G G C C T C –3′ and PvuI B: 5′-C T T C G G T C C T C **C A A T T G** T T G T C A G A A G –3′.

The residues in bold represent the sequences that replaced the original PvuI sites. Several mutagenic primers based on the sequence for *M. sexta* chitinase (Kramer et al., 1993; Genbank accession number U02270 S64757) were utilized in this study, each having the desired substitution as indicated below.

Name	Primer	Amino acid substituted for tryptophan
W145F:	5′-CTAGACCTTGAT**TTC**GAGTACCCAGG-3′	Phe
W145G:	5′-CTAGACCTTGAT**GGG**GAGTACCCAGG-3′	Gly
W145Y:	5′-CTAGACCTTGAT**TAC**GAGTACCCAGG-3′	Tyr
W145H:	5′-CTAGACCTTGAT**CAT**GAGTACCCAGG-3′	His
W145I:	5′-CTAGACCTTGAT**ATC**GAGTACCCAGG-3′	Ile

These primers differed from the wild-type *M. sexta* chitinase cDNA (TGG at W145) in only the residues shown in bold. Mutagenesis was carried out as recommended by the manufacturer and has been described previously (Huang et al., 2000).

The presence of the desired mutations in the resulting mutated plasmids was verified by DNA sequencing. Insert DNAs from these plasmid DNAs containing each of the desired mutations were utilized to generate the baculovirus transfer vectors pVL1393 and the *Autographa californica* nuclear polyhedrosis viruses carrying the mutant *M. sexta* chitinase coding region behind the polyhedrin promoter using the transfer plasmid. Cotransfection of Sf9 cells was carried out with the transfer plasmids and linearized BaculoGold™ DNA transfection kit of Pharmingen Inc. as described in the manufacturer's instructions. The media from co-transfection were used as starting material to obtain high titer virus stocks after amplification in monolayer Sf21 cells using serum-containing medium in 25 cm^2 T-flasks. The presence of the desired mutation in the recombinant viral DNA's was reconfirmed by PCR-amplification of an 800 bp fragment containing the mutated region and flanking sequences and by DNA sequencing of this fragment.

2.2. Expression of mutated proteins in insect cells

Proteins were expressed in the *Trichoplusia ni* cell line, Hi-5, in 75 cm^2 flasks. Hi-5 cells (6×10^6) were

seeded in 75 cm^2 flasks with a total of 15 ml of Excell-405 medium (JRH Biosciences, Lenexa, KS). After allowing the cells to attach for 10 min, baculoviruses containing wild-type or mutated chitinase genes were added to the flasks and incubated at 27°C for five days. The multiplicity of infection used for expression of the proteins was between 3 and 5. Expression level was checked by SDS–PAGE (9% acrylamide) of the proteins in the culture medium followed by staining with Coomassie Blue.

2.3. Purification of wild-type and mutant forms of M. sexta chitinase

The culture medium of Hi-5 cells was collected after five days of infection with the viruses and centrifuged at 1000 g for 10 min. The supernatant (50 ml) was dialyzed against 4 L of 20 mM phosphate buffer, pH 7.0 for one day and loaded on a DEAE-Sepharose (Sigma) (15×20 cm) column equilibrated with 20 mM phosphate buffer, pH 7.0. After loading, the column was washed with 20 ml of 20 mM phosphate buffer, pH 7.0, and eluted with a NaCl gradient (from 0 to 0.35 M) using a total of 140 ml buffer. Fractions of 2 ml were collected and their absorbances were measured at 280 nm. Selected fractions were checked for protein by SDS–PAGE and staining with GELCODE® Blue Stain Reagent (Pierce). Fractions containing the 85 kDa chitinases were pooled, dialyzed and concentrated with a Centriprep® filter (Amicon) using 20 mM phosphate buffer, pH 7.0. The identity of the proteins was confirmed by western blotting using an antibody raised against *M. sexta* chitinase.

2.4. pH-activity profile

The effect of pH on chitinase activity was determined using a polymeric substrate, carboxymethyl Remazol Brilliant Violet chitin (CM-Chitin-RBV, Loewe Biochemica, Nordring, Germany) and an oligosaccharide substrate, 4-methylumbelliferyl β-N, N', N''-triacetylchitotrioside [MU-$(GlcNAc)_3$, Sigma]. For the CM-Chitin-RBV assay, 0.5 μg protein was diluted to 200 μl with water and incubated with 100 μl of CM-Chitin-RBV (2 mg/ml) and 100 μl of 0.2 M universal buffer, pH 4.3–10.3, at 37°C for 2 h. The reaction was stopped by adding 100 μl of 2 M HCl and incubating on ice for 15 min. The reaction mixture was centrifuged at 12,000 g for 5 min and the absorbance of the supernatant was measured at 520 nm. The enzyme activity was expressed as ΔA_{520}/μg/h.

The MU-$(GlcNAc)_3$ assay was conducted according to Hollis et al. (1997) with some modifications. In this assay, 0.1 μg protein was diluted to 33.5 μl with water and incubated with 4 μl of 1.5 mM MU-$(GlcNAc)_3$ and 12.5 μl of 0.2 M universal buffer, pH 4.3–10.3, at 37°C for 15 min. The reaction was stopped by adding 12.5 μl of 2 M HCl. The released methylumbelliferone in each reaction was measured by diluting the reaction mixture with 0.15 M glycine-NaOH buffer (pH 10.5) to 2 ml. Fluorescence spectrophotometry (DyNA Quant 200 Fluorometer, Hoefer) was used to measure the amount of MU with an excitation wavelength of 360 nm and an emission wavelength of 450 nm. A standard curve for MU was made to convert the fluorescent intensity to nmol of product formed. The enzyme activity was expressed as nmol/μg/min.

2.5. pH-stability profile

Protein (0.5 μg in 100 μl of water) was first incubated with 100 μl of 20 mM universal buffer with pH ranging from 3.0 to 10.3 at 4°C for 4 h. Then, the protein was mixed with 100 μl of 2 mg/ml CM-Chitin-RBV in 0.2 M universal buffer at the optimal pH of each enzyme. The reaction was carried out at 37°C for 1 h and stopped by adding 100 μl of 2 M HCl and incubating on ice for 15 min. The absorbance of the mixture was measured at 520 nm after centrifuging the tube at 12,000 g for 5 min. Protein without incubation was used as the control. The activity at each pH relative to the unincubated control was used as the measure of the stability of the enzyme.

2.6. Temperature-activity profile

The effect of temperature on enzyme activity was measured by using CM-Chitin-RBV as substrate. The same amounts of the enzyme, buffer and substrate used for the pH profile of CM-Chitin-RBV were used. The pH of the buffer used for each enzyme was the optimal pH for that enzyme. The temperature range used in the assay was 20–80 °C and the incubation time was 2 h.

2.7. Kinetic assays

Kinetic parameters were obtained by using both CM-Chitin-RBV and MU-$(GlcNAc)_3$ as substrates. In the CM-Chitin-RBV assay, the final volume of the reaction mixture was 400 μl and contained the following: 200 μl of enzyme (0.5 μg), 100 μl of 0.2 M buffer (optimal pH for each enzyme) and 100 μl of CM-Chitin-RBV (ranging from 0.02 mg/ml to 0.3 mg/ml). The reaction was carried out at 37°C for 15 min. The remaining procedure was similar to that used for determination of the pH profile procedure. In MU-$(GlcNAc)_3$ assay, the reaction mixture contained in a total volume of 50 μl: 12.5 μl of 0.2 M universal buffer, pH 6.3, 0.1 μg protein and MU-$(GlcNAc)_3$ ranging from 6 to 300 μM. The rest of the procedure was similar to that for determination of the pH profile. The kinetic parameters of K_m and k_{cat} were obtained by a nonlinear least-square fitting procedure using the ordinary Michaelis–Menten equation and using the plotting software, Kaleidagraph.

2.8. Allosamidin inhibition assay

In the CM-Chitin-RBV assay, different concentrations of allosamidin (5–500 nM) were used in the reaction mixture and the assay was performed exactly as outlined in Section 2.4. The enzyme activity with allosamidin was compared to that of a control without the inhibitor.

2.9. Chitin binding assay

The chitin used for assay was prepared according to Kuranda et al. (1991) and Venegas et al. (1996) with some modifications. Fifty mg of finely ground chitin (crab shell, Sigma) was boiled for 5 min in a 1 ml solution containing 1% SDS and 1% β-mecaptoethanol (β-ME) followed by washing with 15 ml of water. After filtration through 0.45 μm filter, the chitin was suspended in 20 mM phosphate buffer, pH 6.5 containing 0.2% NaN_3. An aliquot of 100 μl of the above suspension was centrifuged and washed twice with the same buffer without 0.2% NaN_3. Two μg of the purified proteins in 150 μl of 20 mM phosphate buffer were added to the chitin pellet. The protein-chitin mixture was incubated at room temperature for 1 h with shaking every 5 min. The mixture was centrifuged at 12,000 g for 10 min and the supernatant was removed as the unbound fraction. The pellet was washed twice with 75 μl of 20 mM sodium phosphate buffer, pH 6.5, each time. The two washes were pooled and saved as the wash fraction. The pellet was finally resuspended with 120 μl of the same buffer plus 30 μl of 5× protein gel loading buffer and boiled for 5 min. After centrifugation, the supernatant was removed and saved as the bound fraction. An aliquot (50 μl) of each fraction was subjected to SDS–PAGE and stained by GELCODE® Blue Stain Reagent.

2.10. Circular dichroism (CD)

CD spectra were obtained with a Jasco J-720 spectropolarimeter at 20°C. Proteins were diluted to around 0.5 μM and prewarmed to room temperature. After degasing, 400 μl of protein was analyzed in the spectropolarimeter and the ellipticity was measured. The exact protein concentrations were measured by the bicinchoninic acid method after CD measurement. The ellipticity was converted to the molar ellipticity.

3. Results

3.1. Characterization of wild-type (W145) and mutant forms (W145I, W145Y, W145F, W145H, and W145G) of insect chitinase

3.1.1. pH optima using an oligosaccharide substrate

The activity of wild-type and mutant forms of *M. sexta* chitinase as a function of pH was determined over the pH range of approximately 2–11 in universal buffer using the oligosaccharide substrate, MU-$(GlcNAc)_3$. The pH-activity profiles of W145I, W145Y, and W145F were similar to that of the wild-type enzyme with a plateau of optimal activity between about pH 5–8. (Fig. 1A). The W145H mutant exhibited a more narrow plateau between pH 5.2–6.2 (Fig. 1B). The histidine mutant was substantially less active at alkaline pH. The W145G mutant did not hydrolyze MU-$(GlcNAc)_3$ over the entire range of pH tested (Fig. 1B). The absence of a side chain group at residue 145 resulted in the loss of activity.

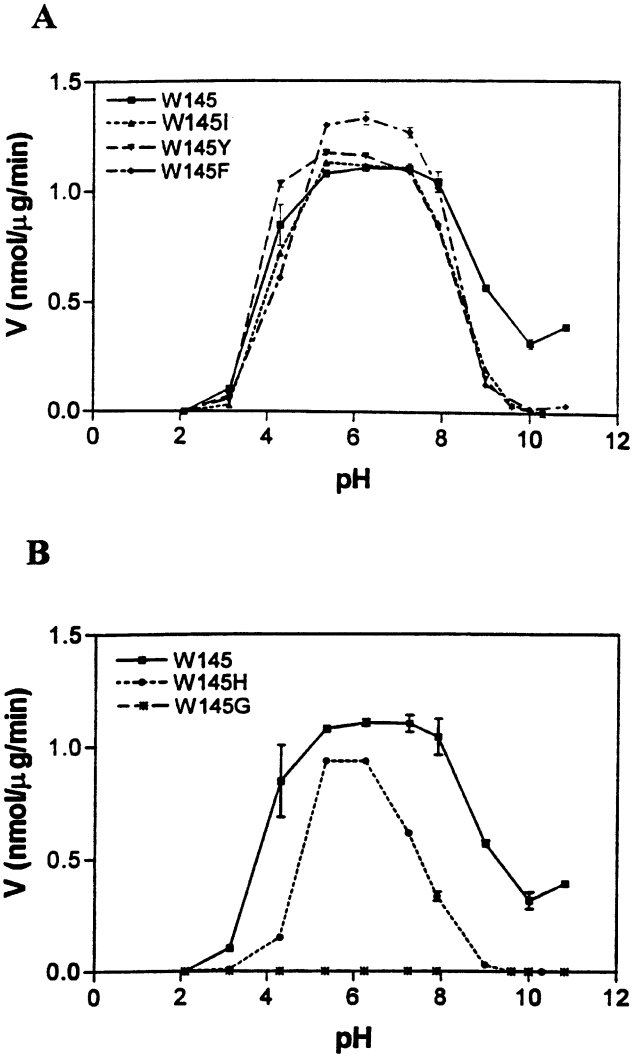

Fig. 1. pH-activity profiles of wild-type and mutant forms of *M. sexta* chitinase using the oligosaccharide substrate, MU-$(GlcNAc)_3$. The activities of wild-type and mutant chitinases were measured using 0.05 μg enzyme mixed with 0.2 mg substrate in 400 μl of 50 mM universal buffer at 37°C for 15 min. (A) Comparison of pH profiles for W145I (▲), W145Y (▼), and W145F (◆) with wild-type chitinase W145 (■). (B) Comparison of pH profiles for W145H (●) and W145G (∗) with wild-type chitinase (■). Standard deviations (S.D.) are shown for each sample as vertical bars above and below the mean values (n=3). In many cases, the vertical bars are not visible due to very small S.Ds.

3.1.2. pH optima using a polymeric substrate

Unlike the results with the oligosaccharide substrate, the pH profiles of the mutants with the polymeric substrate, CM-Chitin-RBV, were significantly different from the wild-type enzyme (Fig. 2). The wild-type enzyme, W145, differed from all of the mutants in two respects. First, it had the highest activity at alkaline pH when compared to the other enzymes. Only this enzyme and W145I had the highest activity at pH values >8 (Fig. 2B). Second, it was active over a broader pH range, extending beyond pH 10. The range of pH optima for the W145F and W145H enzymes was similar to their optimal pH values towards the oligosaccharide substrate, namely pH 5.3–6.8 for W145H and pH 5.3–7.3 for W145F. The W145Y enzyme had a broader pH optimum than W145F and W145H. The highest activity for this enzyme was observed between pH 5.3 and 8.4, with an extended range of high activity by one pH unit towards the alkaline range relative to W145F (Fig. 2A). The profile for the W145I enzyme was similar to W145 up to its optimum of pH 8.0 (Fig. 2B). Beyond this pH, it had a lower activity than the wild-type enzyme. As with the oligomeric substrate, the W145G protein had no detectable activity at any pH tested (Fig. 2B). The optimal pH values for W145 and W145I enzymes were approximately 8.5, and 8.0, respectively. The activities of the three mutant enzymes, W145H, W145F and W145Y, were nearly the same in the pH range 5.4–7.0, 5.4–7.5, and 5.4–8.4, respectively, indicating a broad range of pH optima. All of the mutations exhibited reduced enzymatic activity on the alkaline side of the pH vs activity curve.

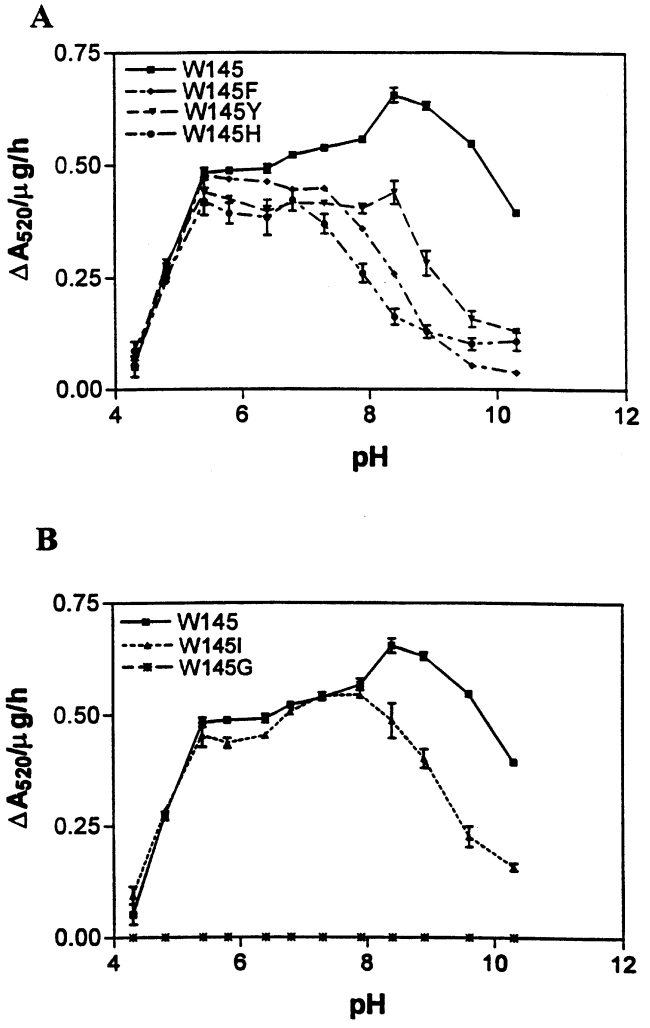

Fig. 2. pH-activity profiles of wild-type and mutant forms of *M. sexta* chitinases using the polymeric substrate, CM-Chitin-RBV. The activities of wild-type and mutant chitinases (1.25 μg/ml) were measured using 0.1 μg enzyme mixed with 0.12 mM substrate in 50 μl of 50 mM universal buffer at 37°C for 2 h. (A) Comparison of pH profile for W145Y (▼), W145F (◆), and W145H (●) with wild-type chitinase W145 (■). (B) Comparison of pH profile for W145I (▲) and W145G (∗) with wild-type chitinase (■). Standard deviations (S.D.) are shown for each sample as vertical bars above and below the mean values (n=3). In many cases, the vertical bars are not visible due to very small S.Ds.

3.2. pH stability of wild-type and mutant insect chitinases using a polymeric substrate

The stability as a function of pH for the W145 enzyme and its mutant forms was tested between pH 3.0 and 10.3 in universal buffer. The activity of each enzyme without preincubation in the buffer at 4°C was defined as 100%. All of the enzymes, including the wild type, were relatively unstable at pH values of 8 and lower (Fig. 3). At higher pH values, the enzymes were more stable. In fact, the wild-type enzyme and W145I had greater activity when preincubated at pH 9.3 or higher. W145H was particularly unstable, losing 50% or more of its activity at all pH values except at pH 10.3. Among the mutants, W145I had the stability curve that most closely resembled that of the wild-type enzyme.

3.3. Temperature-activity profile

The effect of temperature on *M. sexta* chitinase activity was investigated using the polymeric substrate, CM-Chitin-RBV, at temperatures between 20 and 80°C at the optimal pH for each enzyme (pH 8.5 for W145, pH 8.0 for W145I, and pH 5.4 for W145Y, W145F, and W145H). The enzymatic activity at 37°C was defined as 100%. The optimal temperature for W145 and W145I was 37°C (Fig. 4A), but the enzymes retained most of their enzymatic activity even at 49°C. Surprisingly, W145Y and W145F were more active at 49°C than at 37°C (Fig. 4B). Apparently, those mutations increased the thermal stability of the proteins. All of the five enzymes had very low activity at temperatures ≥70°C.

3.4. Inhibition by allosamidin

Allosamidin is a competitive inhibitor of family 18 glycosidases (Sakuda et al., 1987; Koga and Isogai,

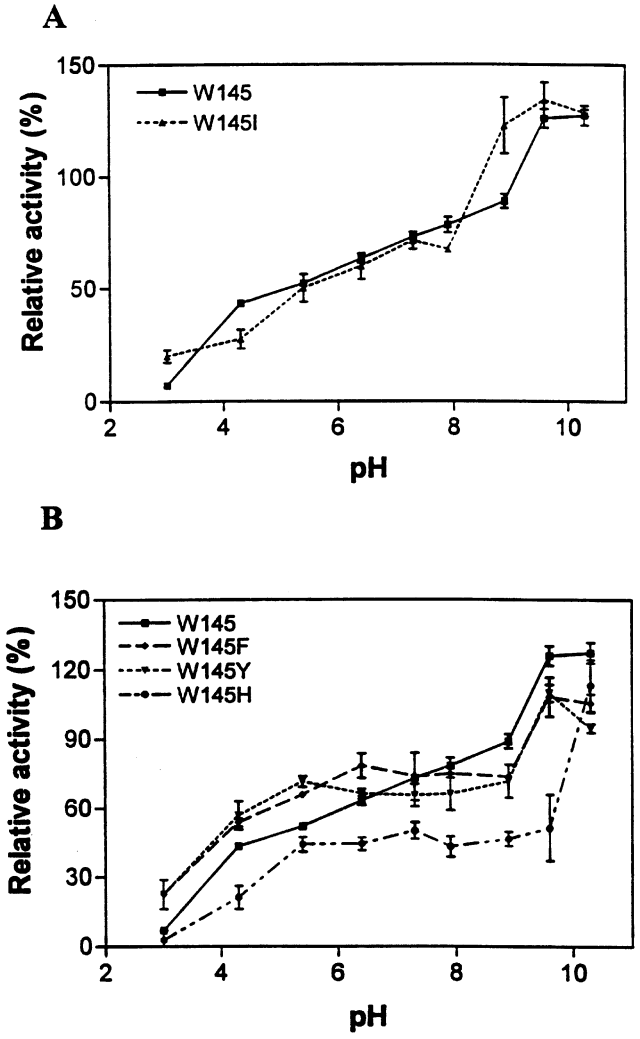

Fig. 3. pH stability-activity profiles of wild-type and mutant forms of *M. sexta* chitinase using the polymeric substrate, CM-Chitin-RBV. The chitinases were incubated in 20 mM universal buffer, pH 3.0–10.3 at 4°C for 4 h. The activity was measured with 0.5 mg/ml CM-Chitin-RBV mixed with 0.5 μg enzyme in 0.2 ml of 10 mM universal buffer at 37°C for 2 h. The remaining activity of the enzyme after each incubation relative to the unincubated enzyme (100%) was used as a measure of pH stability of the enzyme. (A) Comparison of pH stability for W145I (▲) with wild-type chitinase (■). (B). Comparison of pH stability for W145Y (▼), W145H (●), and W145F (◆) with wild-type chitinase W145 (■). Standard deviations (S.D.) are shown for each sample as vertical bars above and below the mean values (n=3). In many cases, the vertical bars are not visible due to very small S.Ds.

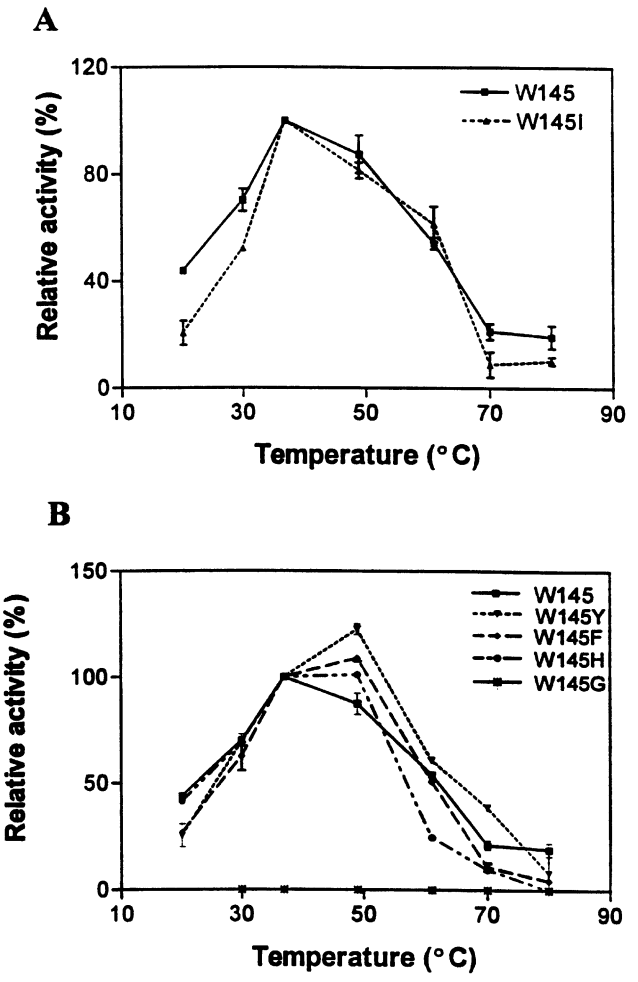

Fig. 4. Temperature-activity profiles for wild-type and mutant *M. sexta* chitinases using the polymeric substrate, CM-Chitin-RBV. The activities of wild-type and mutant chitinases (1.25 μg/ml) were measured with 0.5 mg/ml substrate in 0.2 M universal buffer at the pH optimum for that enzyme for 2 h at temperatures ranging from 20 to 80°C. (A) Comparison of temperature profiles for W145I (▲) with wild-type chitinase (■). (B) Comparison of temperature profiles for W145Y (▼), W145F (◆), W145H (●) and W145G (∗) with wild-type chitinase W145 (■).

1987). Although the inhibition by allosamidin is pH dependent (A/Banat et al. (1999), for greater sensitivity the insect chitinase inhibition assays were done at approximately the optimum pH of each mutant enzyme and the inhibitor concentration was varied from 5 to 500 nM. The reaction without inhibitor was used as the control. The activity of each chitinase at different concentrations of allosamidin relative to the control was plotted against the log of the concentration of the inhibitor. IC_{50} values (the concentration of the inhibitor when the activity was half that of the control) indicated the inhibition efficiency for each enzyme. The IC_{50} value for W145I (15.1 nM) is about the same as that for the W145 enzyme (10.5 nM). This result indicates that W145I can bind to allosamidin nearly as tightly as the wild-type enzyme. The data for the binding of allosamidin by W145Y, W145F, and W145H enzymes as indicated by their IC_{50} values (108, 64 and 99 nM, respectively) indicated that these proteins bind to the inhibitor less tightly at their pH optima. The IC_{50}s were at least six-fold greater than those of W145 and W145I.

3.5. Kinetic assays using oligosaccharide and polymeric substrates

The kinetic parameters, K_m and k_{cat}, were measured by using both the oligosaccharide substrate, MU-$(GlcNAc)_3$, and the polymeric substrate, CM-Chitin-RBV. Table 1A shows that both the K_m and k_{cat} of each mutant towards the oligosaccharide substrate had changed relative to the wild-type enzyme, but that the k_{cat}/K_m ratio remained essentially the same. W145I had the highest k_{cat} and K_m for both the polymeric and the oligomeric substrates, whereas the k_{cat}/K_m of W145I was the lowest for the polymeric substrate (Table 1). W145H had the lowest K_m and k_{cat} for both the polymeric and the oligomeric substrates. The k_{cat}/K_m of W145F, W145Y and W145H were similar to W145 for the polymeric substrate (Table 1B).

3.6. Chitin binding assay

The ability of W145 and the mutant forms to bind to the insoluble substrate, chitin, was studied using a protocol modified from Venegas et al. (1996). Five different mutant forms of chitinase and the wild-type enzyme were tested for their ability to bind to insoluble chitin. The results showed that all of the chitinases associated with chitin. The proteins were present in the pellet fraction, but not in the wash or supernatant fractions (Fig. 5). These results showed that both the wild-type and mutant chitinases including W145G bind to chitin. However, except for the W145G mutant, all of the other four mutants had enzymatic activity comparable to that of the wild-type enzyme. Apparently, W145 is not critical for the binding of the insoluble substrate, but it clearly has a major influence on the enzymatic activity.

3.7. Circular dichroism spectra

The gross structures of wild-type and mutant forms of *M. sexta* chitinase were monitored by circular dichroism (CD). As shown in Fig. 6, the CD spectra of W145, W145F, W145Y, W145I and W145H are similar. However, the spectrum of W145G is quite different from that of W145. This result suggested that the replacement of tryptophan by glycine at this position changed the conformation, whereas none of the other mutations substantially affected the overall structure as measured by CD spectroscopy.

4. Discussion

The conserved region II of chitinases belonging to family 18 glycosyl hydrolases contains several acidic amino acids. The consensus for this region is FDGLDLDWEYP for insects and LDGIDFDIE(L/S)G for plants (Watanabe, 1995). Two interesting observations emerge from the analysis of the sequence variations of several chitinases in this region. The highly conserved residues in this sequence are -DG-D-D-E- (one exception is the fungal *Metarhizium anisopliae* chitinase in which the sequence is VNGFDFDIEVN (protein GenBank ID #CAC07216). The alternating acidic amino acids (ignoring the glycine) are flanked by hydrophobic amino acids. In most cases, they are F, L, V, I, Y, or W. The amino acid separating the two acidic amino acids implicated in catalysis is either W or I (DWE or DIE in the above insect or plant consensus sequences; DYE, DLE, and DFE are occasionally found in other species). The high preference for W in chitinases of animal origin and of I in chitinases of plant and fungal origins suggests some unique roles for these residues in the catalytic site.

Even though there are several studies that have utilized site-directed mutagenesis to investigate the role of the acidic amino acids in conserved region II (Watanabe et al., 1993, 1994; Papanikolau et al., 2001; Thomas et al., 2000; Lin et al., 1999; Lu et al., 2002), there are no studies on the effect of replacing the tryptophan or isoleucine residue sandwiched between the two acidic

Table 1
Kinetic parameters of *M. sexta* chitinases using MU-$(GlcNAc)_3$ (A) and CM-Chitin-RBV (B) and as substrates

Parameter	Enzyme W145	W145I	W145Y	W145F	W145H
(A)					
k_{cat} (s^{-1})	3.3±0.2	7.9±0.5	4.1±0.2	4.8±0.3	3.0±0.1
K_m (μM)	223.4±25.7	486.9±53.2	251.0±25.1	293.9±32.0	186.4±12.7
k_{cat}/K_m (10^4 M^{-1} s^{-1})	1.5	1.6	1.6	1.6	1.6
(B)					
k_{cat} (10^6 $\Delta A_{520} mol^{-1} s^{-1}$)	64.5±3.4	68.5±1.9	58.9±2.1	53.9±2.7	47.6±1.7
K_m (mg/ml)	0.17±0.03	0.23±0.02	0.18±0.02	0.16±0.02	0.14±0.02
k_{cat}/K_m ($10^6 \Delta A_{520} mol^{-1}$ $s^{-1} mg^{-1} ml^{-1}$)	379.5	297.8	327.3	337.2	339.7

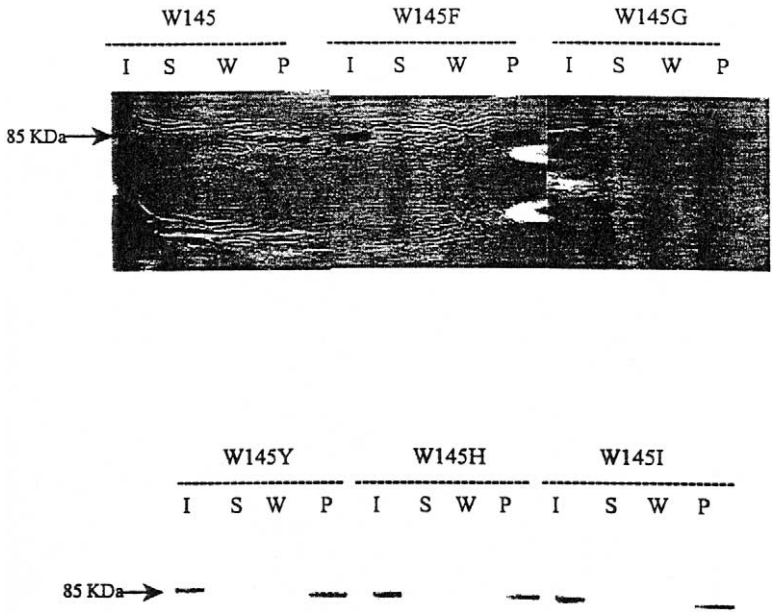

Fig. 5. Chitin binding assay for wild-type and mutant forms of *M. sexta* chitinase as described in the Materials and Methods section. Protein (2.25 μg) was incubated with chitin (50 mg/ml) at room temperature for 1 h. I, initial fraction; S, supernatant (unbound); W, wash fraction; and P, pellet fraction (bound).

amino acids in chitinases of family 18, except for our previous study involving two substitutions at this site (Huang et al., 2000). The latter study indicated that replacement of tryptophan by glycine (W145G) in *M. sexta* chitinase resulted in an enzyme that bound to chitin but was devoid of activity. The W145F mutant, on the other hand, had only about half of the activity of the wild-type enzyme on the polymeric substrate, but it had about the same activity as the wild-type enzyme towards the oligomeric substrate. More importantly, there was a narrowing of the pH range over which the W145F enzyme was active, relative to the wild-type enzyme. These observations prompted the present investigation in which two other hydrophobic amino acids and one polar amino acid, histidine, were substituted for W145 of *M. sexta* chitinase. A comparison of the properties of the homogeneous wild-type enzyme with the five mutant enzymes has led to the elucidation of the role of this tryptophan residue in catalysis.

The most dramatic effect of these substitutions is on the pH-activity profiles. The W145G mutant had no detectable activity, whereas all the other enzymes had substantial activity compared to the wild-type enzyme. A comparison of the pH versus chitinase activity of the wild-type and W145F, W145Y, W145I and W145H mutant proteins with the oligosaccharide substrate, MU-$(GlcNAc)_3$, showed that the activities are relatively similar. On the other hand, with the polymeric substrate, the profiles of activity vs pH were similar in the acidic range but diverged in the alkaline range. Whereas the W145H, W145F and W145Y enzymes had peak activities in the range of pH 5–7, the activities of the wild-type and the W145I enzymes continued to increase with pH and had peak activity between pH 8 and 9. The activities of these two enzymes were substantial even at pH 10 or greater, whereas the other enzymes exhibited much lower activity in the alkaline range. After incubating either W145 or W145I at high pH for several hours, their activities actually increased. We do not have any explanation for why an incubation at high pH would have such an effect. Extending the activity of the enzyme into the alkaline range may be critical for the digestion of chitin by *M. sexta in vivo*, which apparently occurs in the alkaline midgut (pH>10) during the molting process. A chitinase from *B. mori*, an insect with an alkaline midgut similar to *M. sexta*, also has an alkaline optimum pH and a broad range of activity similar to the *M. sexta* enzyme (Koga et al., 1983, 1997). Another chitinase active in the alkaline range is a thermostable chitinase from an alkalophilic *Bacillus* sp. BG-11 (Bhushan, 2000). However, most of the other chitinases of plant, bacterial, and animal origin have pH optima of approximately pH 6. The alkaline pH of the insect midgut may be an adaptation by phytophagous lepidopteran species, which would be expected to be exposed to large amounts of plant chitinases during feeding. The pH optima of plant chitinases are typically in the range of pH 4–6 (Koga et al., 1999), although there are a few exceptions. Therefore, most plant enzymes would be inactive and most pathogens would not grow well in that alkaline pH range where insect chitinases are most active.

The extension of activity of *M. sexta* chitinase towards

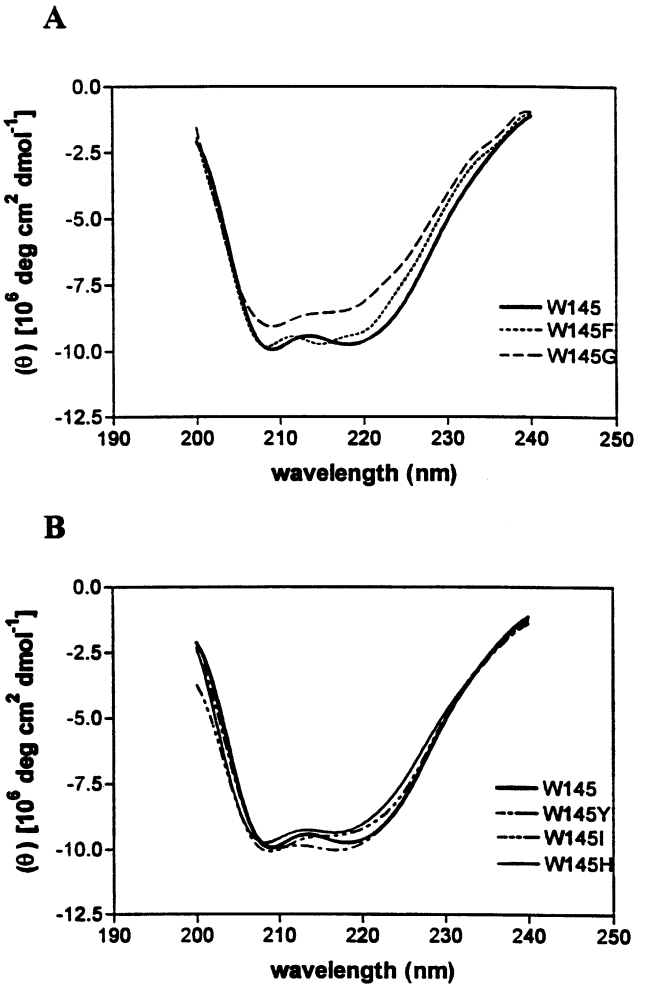

Fig. 6. Circular dichroism (CD) spectra of wild-type and mutant forms of *M. sexta* chitinase. CD of each protein was measured in 20 mM phosphate buffer, pH 6.5 at room temperature in a 0.1 cm cuvette. (A) Comparison of CD spectra for W145F and W145G with that of wild-type chitinase. (B) Comparison of CD spectra for W145Y, W145I, and W145H with that of wild-type chitinase.

the polymeric substrate into the alkaline pH range suggests that W145 in the wild-type enzyme influences the ionization of a group with a pK_a of about 9.5 (see alkaline limb of the activity versus pH curves, Fig. 2). In W145I and W145Y, this group may have a lower pK_a of about 8.5. This pK_a in the W145F enzyme would be slightly lower than that of the W145I and W145Y enzymes. The W145H enzyme exhibited a greater pK_a shift to the acidic side from about pH 9.5 to 7.0. Isoleucine, phenylalanine and tyrosine are hydrophobic amino acids with less hydrophobicity than tryptophan. As reported in W16 mutants of α-galactosidase (Maranville and Zhu, 2000) and W108 mutants in hen's egg white lysozyme (Inoue et al., 1992), the hydrophobic environment of those tryptophan residues altered the pK_a of a neighboring carboxylic group. Histidine is a positively charged residue at pH values ≤7 and may change the polarity of a catalytic residue and make it ionize at a lower pH. However, the hypothetical pK_a for the catalytic residue E146 in the W145H mutant of *M. sexta* chitinase apparently is still higher than the normal pK_a value of approximately pH 4.5 for γ-COOH groups of glutamate residues in proteins. It is possible that another residue(s) contributes to the high pK_a of the catalytic residue. In lysozyme, the electrostatic interaction of D52 with E35 is partly responsible for the abnormal pK_a of E35 of 6.4 (Hashimoto et al., 1996). The D144N mutant of *M. sexta* chitinase apparently has a catalytic residue with a pK_a reduced from the alkaline range to near neutral (Lu et al., 2002) reinforcing the idea that the microenvironment has a strong influence on the ionization of a residue(s) critical for catalysis.

Taken together, the activity profiles of the W145 mutants suggest that the hydrophobicity of a residue at this position raises the pK_a of a neighboring catalytic residue, which is likely to be E146. The E146D mutant of *M. sexta* chitinase is inactive, suggesting that the orientation of the side chain carboxyl group and/or its distance to the susceptible glycosidic bond in the substrate is crucial for catalysis (Lu et al., 2002). The W145G enzyme, which lacks a side chain at position 145, apparently has an altered local structure as revealed by protein modeling (Lu et al., unpublished data). The placement of Gly at position 145 brings D144 and E146 closer together when compared to the wild type enzyme which has a W at that position. This local structural change may contribute to the loss of the enzymatic activity of W145G. The change in CD spectrum of W145G further suggests the importance of the rather bulky W145 side chain in keeping the functional three-dimensional configuration of the catalytic residue(s). Therefore, the alterations in the enzymatic properties observed with these mutant chitinases were apparently caused by changes in the steric configuration of the amino acid at this position and its effect on the surrounding environment of the catalytic residue.

The activity of *M. sexta* chitinase can be influenced not only by pH but also by the composition of the buffer utilized. Wang et al. (1996) reported that, in a glycine-NaOH buffer, the recombinant enzyme has optimal activity at pH 8 with substantially less activity at pH 9. In this study and Huang et al. (2000), a universal buffer composed of acetate, phosphate and borate was used in which the enzyme exhibited greater activity at the high pH values. Apparently, the buffer conditions used in the latter studies activated the enzyme at alkaline pH. The mechanism for this extraordinary behavior is unknown.

Allosamidin is a competitive inhibitor of family 18 glycosyl hydrolases and a few of the family 19 glycosyl hydrolases (Spindler and Spindler-Barth, 1999). It contains two β (1–4) linked N-acetylglucosamine residues and the oxazoline derivative, allosamizoline. It binds to

hevamine at subsites (-3), (-2), and (-1) with the allosamizoline moiety binding to subsite (-1) (van Scheltinga et al., 1994, 1995, 1996). The structure of allosamidin indicates that family 18 chitinases catalyze the degradation of chitin or oligomeric substrates through an oxazoline ion-type of intermediate whose formation is mediated by a substrate-assisted mechanism (van Scheltinga et al., 1994, 1995, 1996; Brameld et al., 1998c). In this mechanism, the acidic residue protonates the glycosidic oxygen and the oxygen of the acetamido carbonyl group of GlcNAc attacks the C1′ of the sugar to form the oxazoline intermediate (Piszkiewiez and Bruice, 1967, 1968). The substrate-assisted mechanism involves only one acidic amino acid residue in the catalysis. This mechanism also involves a double displacement hydrolysis, which results in the retention of the anomeric configuration (β-anomer) of the glycosidic bond that is hydrolyzed. The retention mechanism has been determined for the following family 18 chitinases: a plant chitinase hevamine from *H. brasiliensis* (van Scheltinga et al., 1995), a bacterial chitinase from *B. circulans* WL-12 (Armand et al., 1994), a fungal chitinase from *C. immitis* (Fukamizo et al., 2001), and a chitinase from the silkworm, *Bombyx mori* (A/Banat et al., 1999). *M. sexta* chitinase, a member of family 18 hydrolases, also is inhibited by allosamidin. The binding of *M. sexta* chitinase to allosamidin is even stronger than the binding of other chitinases (Spindler and Spindler-Barth, 1999). We propose that *M. sexta* chitinase also uses a retaining mechanism in the hydrolysis of N-acetylchitooligosaccharides and chitin. All of the W145 mutants were inhibited by allosamidin, indicating that the substitution of W145 did not alter the catalytic mechanism.

Tryptophan is involved in interactions with sugars by stacking interactions and hydrogen bonding (Vyas, 1991). W255 of hevamine, a plant chitinase (van Scheltinga et al., 1995), and W62 of chicken lysozyme (Maenaka et al., 1998) are involved in substrate binding by stacking interactions and as members of a hydrogen-bonding network with the substrates. W145I in *Manduca* chitinase, which lacks the ability to form a hydrogen bond and to stack with a sugar, has the highest K_m towards the polymeric substrates. However, the K_m difference between W145I and W145 is only about 30%. The K_m's of W145F and W145Y towards the polymeric substrate are close to that of the W145 enzyme. The K_m of W145H is slightly lower than that of W145. These results suggest that W145 of *M. sexta* chitinase is not essential for polymeric substrate binding. The k_{cat} values of the W145 mutants were not significantly different from that of W145. Therefore, W145 is also not an essential amino acid for catalysis. For the oligomeric substrate, both the K_m and k_{cat} of W145 mutants are slightly different from those of W145 with W145I showing the highest value (two-fold greater). However, the k_{cat}/K_m of W145 mutants are nearly the same as that of W145. Therefore, the overall catalytic efficiencies of the W145 mutants are similar to that of W145.

Instead of influencing the ionization of a catalytic group as demonstrated in this study of *M. sexta* chitinase, some tryptophan residues in other chitinolytic enzymes are involved in substrate binding interactions. Two tryptophans in *B. circulans* A1 chitinase and three in *Serratia marcescens* 2170 chitinase apparently help to guide chitin chains into the catalytic clefts of these enzymes during the hydrolysis of crystalline chitin (Uchiyama et al., 2001). The activity of the latter's mutant enzymes where alanine was substituted for those conserved tryptophans towards soluble substrates, however, was unaffected. Perrakis et al. (1994) identified several conserved tryptophans in the ChiA N-domain of *S. marcescens*, which are located in ideal positions to facilitate interactions with an extended sugar chain modeled in the catalytic groove that is found in a separate domain. There are several tryptophans in *M. sexta* chitinase, which probably have comparable roles in substrate stacking and hydrogen-bonding interactions (Zhu, 1998). Those aromatic residues in insect chitinase will be the subject of a future site-directed mutagenesis study.

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